



Urine Human Chorionic Gonadotropin (hCG) Assay Development Report

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September 28, 2012

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1. ASSAY INFORMATION [TC "ASSAY INFORMATION" \f C \l "2"]

1.1 Assay Specifications [TC "Assay Specifications" \f C \l "3"]

Human chorionic gonadotropin (hCG) is a glycoprotein hormone normally produced by the placenta during pregnancy. The hCG molecule (MW: 37,500) consists of alpha and beta subunits. The alpha subunit (MW: 14,000) is identical to the alpha subunit of the pituitary glycoprotein hormones: luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). The beta subunit (MW: 23,500) confers biological and immunological specificity to the entire hCG molecule¹.

This assay is designed to quantitatively determine the level of hCG in human urine.

1.2 Reference Assays [TC "Reference Assays and Standards" \f C \l "3"]

DRG One Step HCG Pregnancy Test (Cat# RAP-2818, certified for diagnostic use) has been used to verify pregnancy clinical samples.

1.3 Materials and Methods [TC "Materials and Methods" \f C \l "1"]

A biotin-labeled anti-hCG antibody coated on an avidin surface serves as the capture surface for the sandwich ELISA. The urine sample is incubated on the capture surface, then an alkaline phosphatase-labeled anti-hCG antibody is incubated on the surface. After the detection antibody incubation, the surface is washed and the alkaline phosphatase substrate is incubated on the surface, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Human chorionic gonadotropin (hCG)	Abazyme	HOR-250
Human chorionic gonadotropin (hCG) Beta	Genway	GWB-5D4D09
Mouse Anti-hCG beta(HCG-61) (CAb)	Abcam	ab764
Mouse Anti-hCG beta (BC152) (DAb)	Abcam	ab9229
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13
Biotin Labeling Kit (SH)	Dojindo	LK10
Phospho Glo Substrate	KPL	55-60-04
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G

2. ASSAY DEVELOPMENT [TC "ASSAY OPTIMIZATION" \F C \L "2"]

2.1 Capture and Detection Antibody Information

The capture and detection antibody pair was selected based on Serum hCG Assay developed for Theranos 3.0 System previously.

Table [SEQ Table * ARABIC]: Capture and Detection Antibody Information

Antibody Type	Antibody Description	Vendor	Cat #
Capture Antibody (CAb)	Mouse Anti-hCG beta(HCG-61)	Abcam	ab764
Detection Antibody (DAb)	Mouse Anti-hCG beta (BC152)	Abcam	ab9229

2.2 Alkaline Phosphatase Stabilizers

Stabilzyme AP Stabilizer was selected as detection antibody diluent with the DAb concentration at 100 ng/mL based on Serum hCG Assay developed for Theranos 3.0 System previously.

2.3 Diluent Test

3% BSA in TBS Blocking Buffer and Starting Block were tested as sample diluent. Starting Block did not show any advantage over 3% BSA in TBS Blocking Buffer. Therefore, 3% BSA in TBS Blocking Buffer was used as the sample diluent for Theranos urine hCG assay development.

Table [SEQ Table * ARABIC]: Diluent Test (Protocol: Generic 2_25x_5_5_5)

Nominal [hCG] mIU/mL	3% BSA blocking buffer			Starting Block		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
100000	3093494	4	2864	3338293	7	2177
10000	2473030	4	2290	2326447	5	1517
1000	308814	21	286	271041	25	177
100	19008	18	18	18335	19	12
10	2997	10	3	3618	12	2
0	1080	15	1	1534	27	1

2.4 Sample Dilution Test

The effect of sample dilution was tested with final sample dilution factors of 1:25, 1:50, and 1:100 into 3% BSA in TBS blocking buffer. When the sample dilution is at 1:50, the signal modulation was the best.

Table [SEQ Table * ARABIC]: Sample Dilution Test

Nominal [hCG] mIU/mL	Assigned to CLIA mIU/mL	Generic2_25x_5_5_5			Generic2_25x_5_5_5*			Generic2_100x_5_5_5		
		Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
100000	185560.0	2836575	7	1715	2872923	7	1960	2801031	7	1725
10000	18556.0	2072481	12	1253	1620809	14	1106	870080	13	536
1000	1855.6	255795	16	155	93423	9	64	29745	8	18
100	185.6	8490	7	5	4435	21	3	2441	8	2
50	92.8	2934	4	2	3326	23	2	2115	4	1
25	46.4	26	58	0	2368	10	2	5232	15	3
10	18.6	1814	23	1	1807	16	1	1547	7	1
0	0.0	1654	11	1	1466	6	1	1624	9	1

* Actual sample dilution is 1:50 (1: 2 sample predilution first, then run Generic 2_25x_5_5_5 protocol)

2.5 Coincubation Test

Coincubation test had been done to compare with the current protocol Generic2_25x_5_5_5. It showed that Generic2_25x_coincubation_5_5 reduced saturation at the top point as well as background. Therefore, coincubation protocol was used for the further assay development.

Table [SEQ Table * ARABIC]: Coincubation Test

Normal [hCG] mIU/mL	Generic2_25x_5_5_5			Generic2_25x_coincubation_5_5		
	Mean RLU	CV%	Modulation	Mean RLU	CV%	Modulation
100000	3093494	4	2864	804666	8	913
10000	2473030	4	2290	583334	8	662
1000	308814	21	286	114965	25	130
100	19008	18	18	11607	15	13
10	2997	10	3	3277	16	4
0	1080	15	1	881	8	1

Since the sample dilution test with protocol Generic25x_5_5_5 showed that 1:50 sample dilution is better. Therefore, we also tested 1:50 sample dilution with coincubation protocol. However, the top point became saturated and the signal modulation at 10 mIU/mL and 100 mIU/mL was reduced. Since the sensitivity at the low end of the assay is important, the protocol Generic2_25x_coincubation_5_5_5 was selected as final protocol.

Table [SEQ Table * ARABIC]: Coincubation Test with sample dilution at 1:50

Normal [hCG] mIU/mL	Assigned to CLIA mIU/mL	Generic2_25x_5_5_5 *		
		Mean RLU	CV%	Modulation
100000	185560.0	1141040	6	5068
10000	18556.0	481893	28	2140
1000	1855.6	36961	10	164
100	185.6	1812	15	8
50	92.8	717	10	3
25	46.4	458	11	2
10	18.6	379	16	2
0	0.0	225	16	1

* Actual sample dilution is 1:50 (1:2 sample predilution and run Generic 2_25x_5_5_5 protocol)

2.6 Intact hCG and Free hCG Beta Calibrator Comparison

Since a large amount of free hCG subunits and partially degraded forms of hCG are in the urine due to degradation, the intact hCG and free hCG beta calibration had been compared using final protocol Generic2_25x_coincubation_5_5. The result showed that dose response of intact hCG and free hCG beta tracked very well.

Table [SEQ Table * ARABIC]: Intact hCG calibrator

[hCG] mIU/mL	Mean RLU	CV%	Modulation
100000	1055941	1	4513
10000	600131	21	2565
1000	93273	14	399
100	6279	23	27
50	1716	16	7
25	629	24	3
10	446	18	2
0	234	24	1

Table [SEQ Table * ARABIC]: Free hCG Beta calibrator

[free hCG beta] ng/mL*	Mean RLU	CV%	Modulation
10000	740391	5	3095
1000	518038	6	2165
100	106948	16	447
10	27377	26	114
5	10016	11	42
2.5	4026	20	17
1	1580	7	7
0	239	15	1

(*note: intact hCG calibrator was converted to ng/mL, then use the same concentration for free hCG beta)
 (1ug=9.28 IU based on WHO 1st IRP/3rd IS 75/537)

Figure [SEQ Figure * ARABIC]: Dose Response: Intact hCG vs Free hCG Beta calibrator
[SHAPE * MERGEFORMAT]

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2.7 Clinical Urine Samples

To further confirm that intact hCG and free hCG Beta calibration tracked well, more calibrator points had been added to the calibration. The results showed that intact hCG and free hCG dose response tracked well. Further, 16 clinical urine samples had been tested on Theranos 3.0 System and calculated with both intact hCG and free hCG beta standard curves. Meanwhile, the clinical urine samples were tested for pregnancy using DRG One Step HCG Pregnancy Test. The clinical samples were sent to CLIA lab for hCG test using Siemens Immulite 2000 instrument (For Research Use only, not validated). For further development, only intact hCG calibration would be used.

Table [SEQ Table * ARABIC]: Intact hCG Calibration

[hCG] conversion	[hCG]	Signal, RLU	Back-Calculated Conc., mIU/mL
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to ng/mL*	mIU/mL	Mean RLU	CV%	Mean RLU	CV%	%Recovery
10000	100000	1055941	1	OORH		
1000	10000	533618	10	10927	19.1	109
550.0	5500	359626	14	5306	23.1	96
100	1000	93273	14	866	13.7	87
10	100	6279	23	135	13.9	135
5.0	50	1012	21	35	20.8	71
2.5	25	629	24	21	28.8	84
1	10	446	18	14	25.2	136
0.0	0	263	31	OORL		

*note: intact hCG calibrator was converted to ng/mL (1ug=9.28 IU based on WHO 1st P/3rd IS 75/537)

Figure [SEQ Figure * ARABIC]: Intact hCG Dose Response

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: hCG test result for clinical samples

[SHAPE * MERGEFORMAT]

Table [SEQ Table * ARABIC]: Free hCG Beta Calibration

Nominal [Free hCG beta] ng/mL	CLIA Result mIU/mL	Converted to mIU/mL (based on CLIA result)	Signal, RLU		Back-Calculated Conc., mIU/mL		
			Mean RLU	CV%	Mean RLU	CV%	%Recovery
10000	42230	161320	740391	5	OORH		
1000	4578	16132	518038	6	OORH		
550.0		8873	606640	22	9139	13.8	103
100	680	1613	106948	16	1661	19.6	103
55		887	46214	9	567	12.4	64
10.0	163	161	27377	26	288	34.3	179
5		81	10016	11	82	12.9	102
2.5	78.1	40	4026	20	33	17.3	81
1.8		28	1893	15	19	7.4	68
1	15.7	16	1580	7	OORL		
0	<1.00	0	239	15	OORL		

Note: CLIA Lab used Siemens Immulite 2000 for urine hCG test

Figure [SEQ Figure * ARABIC]: Free hCG Beta Dose Response

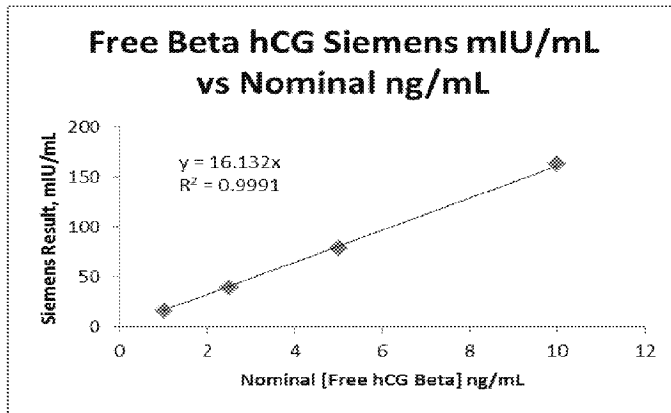


Figure [SEQ Figure * ARABIC]: Free hCG Beta Dose Response

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Free hCG Beta Dose Response

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: hCG Dose Response

[SHAPE * MERGEFORMAT]

Table [SEQ Table * ARABIC]: hCG Test Result for Clinical Samples

Sample Type	Sample ID	DRG Prgnancy Test	CLIA, mIU/mL	Theranos Result, mIU/mL		Pregnancy Weeks
				Intact hCG Calculation	Free Beta hCG Calculation	
Negative Control	CN	Negative	1.68	OORL	OORL	N/A
Positive Control	CP	Positive	240	157	65	Unknown
Negatives	N01	Negative	1.43	OORL	OORL	N/A
	N02	Negative	< 1.00	OORL	OORL	N/A
	N03	Negative	< 1.00	OORL	OORL	N/A
	N04	Negative	1.12	OORL	OORL	N/A
Positives	P01	Positive	44902	OORH	OORH	12
	P02	Positive	4479	1643	2687	36
	P03	Positive	196188	OORH	OORH	14
	P04	Positive	7330	603	861	20
	P05	Positive	9451	OORH	OORH	27
	P06	Positive	19532	OORH	OORH	35
	P07	Positive	2678	546	743	15
	P08	Positive	17616	OORH	OORH	21
	P09	Positive	21890	OORH	OORH	26
	P10	Positive	9164	OORH	OORH	22

* These samples run as 1:100 preclution in Siemens Immulite 2000

2.8 Clinical Samples Pre-dilution Test

Since most of the clinical urine samples tested are out of range high on Theranos and need dilution for Siemens instrument from CLIA Lab, sample predilution has been tested. Clinical urine samples P1-P10 were diluted at 1:50 or 1:100 first, then tested for hCG on Theranos and Siemens Immulite. A new intact hCG calibration had been done with more calibrator points for calculation.

Table [SEQ Table * ARABIC]: Intact hCG Calibration

[hCG] mIU/mL	Signal, RLU		Back-Calculated Conc., mIU/mL		
	Mean RLU	CV%	Mean RLU	CV%	%Recovery
100000	1034297	17	OORH		
10000	511029	8	9782	7	98
5500	252720	8	5493	6	100
1000	24245	10	1075	7	107
550	7345	15	459	12	84
100	1580	12	121	12	121
50	605	15	42	18	84
25	428	18	27	24	108
17.5	311	6	OORL		
10	297	16	OORL		
0	210	25	OORL		

Figure [SEQ Figure * ARABIC]: Intact hCG Standard Curve

[SHAPE * MERGEFORMAT]

Table [SEQ Table * ARABIC]: Clinical Urine Pre-dilution Test

Sample ID	Dilution*	Siemens, mIU/mL	Theranos, mIU/mL
DP1	1:50	790	365
DP2	1:50	36.9	25

DP3	1:50	3395	1127
DP4	1:50	167	133
DP5	1:50	138	190
DP6	100	125	280
DP7	100	32.2	13
DP8	100	77.4	61
DP9	100	108	75
DP10	100	69	53

* Sample DP1-DP10 are the samples that had been diluted from original samples P1-P10 respectively.

Figure [SEQ Figure * ARABIC]: Siemens vs Theranos Results for Pre-diluted Clinical Urine Samples

[SHAPE * MERGEFORMAT]

2.9 Determination of Expected LLOQ and ULOQ

A lot of reagents were produced and a calibration was performed on the final assay conditions of 100 ng/mL Dab in Stabilzyme AP Stabilizer, 5 ug/mL capture antibody with UA coat, and a 1:25 sample dilution with 3 cartridges per point. The protocol for Theranos 3.0 System is Generic2_25x_coincubationx_5_5.

Dexter was run to decide the LLOQ and ULOQ. The LLOQ was 10 mIU/mL, and the ULOQ was 10000 mIU/mL. A standard curve was used to calibrate the Theranos System from 10 to 10000 mIU/mL.

Table [SEQ Table * ARABIC]: Intact hCG Calibration

[hCG] mIU/mL	Signal, RLU		Back-Calculated Conc., mIU/mL		
	Mean RLU	CV%	Mean RLU	CV%	%Recovery

100000	1066079	8	OORH		
10000	590377	9	10112	12	101
1000	30083	10	992	5	99
100	1163	22	108	24	108
50	608	11	46	16	92
25	404	12	24	20	97
10	259	12	11	23	106
0	206	15	OORL		

$$=10^{((0.1365)*(\text{LOG}(S))^3-1.7816*(\text{LOG}(S))^2+8.2272*(\text{LOG}(S))-10.376)}$$

Figure [SEQ Figure * ARABIC]: Intact hCG Standard Curve

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Standard Curve for Determination of LLOQ and ULOQ on Dexter

[SHAPE * MERGEFORMAT]

2.10 Early Pregnancy Urine Samples

46 Early Pregnancy Urine Samples were obtained from ZeptoMetrix and Quest Diagnostics. These clinical samples were tested on DRG Pregnancy Test kit to confirm pregnancy. These samples were then tested on Theranos 3.0 system with final protocol: Generic2_25x_coincubationx_5_5.



The Theranos urine hCG assay cutoff is 25 mIU/mL (hCG concentration in urine ≥ 25 mIU/mL is positive for Pregnancy). In general, Theranos urine hCG assay correlated very well to DRG Pregnancy Test except that 3 out of 46 urine samples didn't show the correlation.

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Table [SEQ Table * ARABIC]: Early Pregnancy Urine Samples Correlation

Sample	DRG Pregnancy Test	CLIA Lab Immulite		Theranos (intact hCG)	Pregnancy Weeks
		Results (mIU/mL)	Dilutions		
E1	Positive	52288	100	OORH	5
E2	Positive	2234	1	5279	5
E3	Positive	13384	100	8892	7
E4	Positive	2213	1	547	3
E5	Positive	68042	100	OORH	7
E6	Positive	2240	1	6223	7
E7	Positive	29253	100	OORH	6
E8	Positive	86597	100	OORH	7
E9	Positive	85045	100	OORH	5
E10	Positive	115229	100	OORH	7
E11	Positive	4344	1	1484	7
E12	Positive	47860	100	OORH	7
E13	Positive	325	1	636	3
E14	Positive	43336	100	OORH	3
E15	Positive	198551	100	OORH	7
E16	Positive	310864	100	OORH	7
E17	Positive	28864	100	OORH	7
E18	Positive	53733	100	OORH	7
E19	Positive	51594	100	OORH	7
E20	Positive	3318	1	OORH	6
E21	Negative	22.6	1	23	7
E22	Positive	4820	100	9580	5
E23	Positive	6695	100	7484	5
E24	Positive	92875	100	OORH	7
E25	Positive	547	1	639	5
E26	Positive	3635	100	6076.70	3
E27	Weak Positive	345	1	52.31	3
E28	Positive	5862	100	8473.75	3
Q1	Positive	4697	1	1764.73	3
Q2	Positive	34597	100	3431.23	3
Q3	Positive	124	1	25.98	3
Q4	Negative	10.1	1	OORL	1 to 2
Q6	Positive	145	1	OORL	4
Q7	Positive	730	1	182.64	4
Q8	Positive	2025	100	1150.93	4
Q9	Positive	1162	100	4459.53	3
Q10	Weak Positive	133	1	OORL	4
Q11	Positive	1372	1	584.55	3
Q12	Positive	538	1	42.40	4
Q13	Positive	1753	100	536.28	3
Q14	Positive	4855	1	85.26	4
Q16	Positive	12904	100	10789.15	4
Q17	Weak Positive	339706	100	OORH	4
Q18	Weak Positive	16024	100	OORH	4
Q19	Weak Positive	3078	100	268.42	4
Q20	Negative	250	1	261.36	4
Positive					
Negative					

References

1. Stenman, U. Human Chorionic Gonadotropin in Cancer. *Clinical Biochemistry* 2004; 37: 549-561.

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